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(54) A NEW GENE OF HUMAN LYSOENZYME, THE ENCODING POLYPEPTIDE AND METHODS FOR PREPARING THEM

GEN EINES MENSCHLICHEN LYSOZYMS, DAVON KODIERTES POLYPEPTID UND VERFAHREN ZU DEREN HERSTELLUNG

GENE DE LYSOZYME HUMAIN, POLYPEPTIDE CODE PAR CELUI-CI ET LEUR PROCEDE DE PREPARATION

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(73) Proprietor: **Yu, Long**
Shanghai, 200433 (CN)

(72) Inventors:
- **Fu, Qiang,**
  Shanghai 200433 (CN)
- **Yong, Zhao,**
  Shanghai, 200433 (CN)
- **Zhang, Honglai,**
  Shanghai 200433 (CN)
- **Bi, Anding,**
  Shanghai 200433 (CN)

(74) Representative: **Viering, Jentschura & Partner**
Postfach 22 14 43
80504 München (DE)

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Remarks:
The file contains technical information submitted after the application was filed and not included in this specification

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Description

Field of invention

[0001] The invention relates to a new polynucleotide, the polypeptide encoded by said polynucleotide, the uses of said polynucleotide and polypeptide, and the methods for preparing same. In particular, the polypeptide of the invention is identified as a new member of the lysozyme family.

Prior art

[0002] Lysozyme exists ubiquitously in all parts of organisms, including various tissues, organs, and sera; it is especially abundant in egg white. Lysozyme is mainly secreted by the epithelial cell of certain glands and some kinds of leukocyte.

[0003] Lysozyme was first reported by Fleming, et al. in 1922. Afterward, lysozyme has been widely studied. A lot of papers concerning its crystal structure, protein catalytic domains, catalytic dynamics, immunology, molecular evolutionary, and so on, have been published, Lysozyme is one of the proteins that are studied most extensively and intensively. However, the study on lysozyme gene is not yet sufficient. Nowadays, only a few lysozyme genes from different species, such as E.coli T4, salmonella P22 phage, bacillus ϕ phage and chicken, etc., have been cloned. (1983 J. Mol. Biol. 165. 229-248; 1985 Virology 143, 280-289; 1987 Proc. Natl. Acad. Sci. USA, 77, 5759-5763). The cloning about human lysozyme gene was also reported (1988, Gene 66,223-234).

[0004] The main function of lysozyme is to hydrolyze the beta(1-4) glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylgluconic acid (NAG) of the bacterial cell wall. In the organism, lysozyme can act as a nonspecific immune molecule against bacterial infections, and as a digestive enzyme in enteron and some mollusks which live on bacteria. Further, lysozyme has the function of inhibiting tumor growth. Therefore, lysozyme has important applications in both industry and medicine.

Summary of Invention

[0005] One purpose of the invention is to provide a new polynucleotide which encodes a new member of lysozyme gene family. The new human lysozyme is named LYC4.

[0006] Another purpose of the invention is to provide a new member of lysozyme protein family, which is named LYC4.

[0007] Still another purpose of the invention is to provide a new method for preparing said new human lysozyme by recombinant techniques.

[0008] The invention also relates to the uses of said human lysozyme and its coding sequence.

[0009] In one aspect, the invention provides an isolated DNA molecule, which comprises a nucleotide sequence encoding a polypeptide having human LYC4 protein activity, wherein said nucleotide sequence shares at least 70% homology to the nucleotide sequence of nucleotides 179-619 in SEQ ID NO: 3, or said nucleotide sequence can hybridize to the nucleotide sequence of nucleotides 179-619 in SEQ ID NO: 3 under moderate stringency. Preferably, said nucleotide sequence encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 4 or of 20-146 in SEQ ID NO: 4. More preferably, the sequence comprises the nucleotide sequence of nucleotides 179-619 in SEQ ID NO: 3.

[0010] Further, the invention provides an isolated LYC4 polypeptide, which comprises a polypeptide having the amino acid sequence of SEQ ID NO: 4 or of 20-146 in SEQ ID NO: 4, its active fragments, and its active derivatives. Preferably, the polypeptide is a polypeptide having the amino acid sequence of SEQ ID NO: 4 or of 20-146 in SEQ ID NO: 4.

[0011] The invention also provides a vector comprising said isolated DNA.

[0012] The invention further provides a host cell transformed with said vector.

[0013] In another aspect, the invention provides a method for producing a polypeptide with the activity of LYC4 protein, which comprises:

(a) forming an expression vector of LYC4 protein comprising the nucleotide sequence encoding the polypeptide having the activity of LYC4 protein, wherein said nucleotide sequence is operably linked with an expression regulatory sequences, and said nucleotide sequence shares at least 70% homology to the nucleotide sequence of positions 179-619 in SEQ ID NO: 3;
(b) introducing the vector of step (a) into a host cell, thereby forming a recombinant cell of LYC4 protein;
(c) culturing the recombinant cell of step (b) under the conditions suitable for the expression of LYC4 polypeptides;
(d) isolating the polypeptides having the activity of LYC4 protein.

[0014] In one embodiment of the present invention, the isolated polynucleotide has a full length of 775 nucleotides, whose detailed sequence is shown in SEQ ID NO: 3. The open reading frame (ORF) locates at nucleotides 179-619,

[0015] In the present invention, the term "isolated" or "purified" or "substantially pure" DNA refers to a DNA or fragment

which has been isolated from the sequences which frank it in a naturally occurring state. The term also applied to DNA or DNA fragment which has been isolated from other components naturally accompanying the nucleic acid and from proteins naturally accompanying it in the cell.

[0016] In the present invention, the term "LYC4 protein encoding sequence" or " LYC4 polypeptide encoding sequence " refers to a nucleotide sequence encoding a polypeptide having the activity of LYC4 protein, such as the nucleotide sequence of positions 179-619 in SEQ ID NO: 3 or its degenerate sequence. The degenerate sequences refer to the sequences formed by replacing one or more codons in the ORF of 179-619 in SEQ ID NO: 3 with degenerate codes which encode the same amino acid. Because of the degeneracy of codon, the sequence having a homology as low as about 70% to the sequence of nucleotides 179-619 in SEQ ID NO: 3 can also encode the sequence shown in SEQ ID NO: 4. The term also refers to the nucleotide sequences that hybridize with the nucleotide sequence of nucleotides 179-619 in SEQ ID NO: 3 under moderate stringency or preferably under high stringency. In addition, the term also refers to the sequences having a homology at least 70%, preferably 80%, more preferably 90% to the nucleotide sequence of nucleotides 179-619 in SEQ ID NO: 3. Moreover, the term includes a nucleotide sequence encoding a mature protein without signal peptide, such as the nucleotide sequence of position 236-619 in SEQ ID NO: 3.

[0017] The term also refers to variants of the sequence in SEQ ID NO: 3, which are capable of coding for a protein having the same function as human LYC4 protein. These variants includes, but are not limited to: deletions, insertions and/or substitutions of several nucleotides (typically 1-90, preferably 1-60, more preferably 1-20, and most preferably 1-10) and additions of several nucleotides (typically less than 60, preferably 30, more preferably 10, most preferably 5) at 5' end and/or 3' end.

[0018] In the present invention, "substantially pure" proteins or polypeptides refers to those which occupy at least 20%, preferably at least 50%, more preferably at least 80%, most preferably at least 90% of the total sample material (by wet weight or dry weight). Purity can be measured by any appropriate method, e.g., in the case of polypeptides by column chromatography, PAGE or HPLC analysis. A substantially purified polypeptides is essentially free of naturally associated components.

[0019] In the present invention, the term "LYC4 polypeptide "or "LYC4 protein " refers to a polypeptide having the activity of LYC4 protein comprising the amino acid sequence of SEQ ID NO: 4 or of positions 20-146 of SEQ ID NO: 4. The term also comprises the variants of said amino acid sequence which have the same function of human lysozyme. These variants include, but are not limited to, deletions, insertions and/or substitutions of several amino acids (typically 1-50, preferably 1-30, more preferably 1-20, most preferably 1-10), and addition of one or more amino acids (typically less than 20, preferably less than 10, more preferably less than 5) at C-terminal and/or N-terminal. For example, the protein function are usually unchanged when an amino residue is substituted by a similar or analogous one. Further, the addition of one or several amino acids at C-terminal and/or N-terminal will not change the function of protein. The term also includes the active fragments and derivatives of LYC4 protein.

[0020] The variants of polypeptide include homologous sequences, allelic variants, natural mutants, induced mutants, proteins encoded by DNA which hybridizes to LYC4 DNA under high or low stringency conditions as well as the polypeptides or proteins retrieved by antisera raised against LYC4 polypeptide. The present invention also provides other polypeptides, e.g., fusion proteins, which include the LYC4 polypeptide or fragments thereof. In addition to substantially full-length polypeptide, the soluble fragments of LYC4 polypeptide are also provided. Generally, these fragments comprise at least 10, typically at least 30, preferably at least 50, more preferably at least 80, most preferably at least 100 consecutive amino acids of human LYC4 polypeptide.

[0021] The present invention also provides the analogues of LYC4 protein or polypeptide. Analogues can differ from naturally occurring LYC4 polypeptide by amino acid sequence differences or by modifications which do not affect the sequence, or by both. These polypeptides include genetic variants, both natural and induced. Induced variants can be made by various techniques, e.g., by random mutagenesis using irradiation or exposure to mutagens, or by site-directed mutagenesis or other known molecular biologic techniques. Also included are analogues which include residues other than those naturally occurring L-amino acids (e.g., D-amino acids) or non-naturally occurring or synthetic amino acids (e.g., beta- or gamma-amino acids). It is understood that the polypeptides of the invention are not limited to the representative polypeptides listed hereinabove.

[0022] Modifications (which do not normally alter primary sequence) include in vivo, or in vitro chemical derivation of polypeptides, e.g., acelylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in the further processing steps, e.g., by exposing the polypeptide to enzymes which affect glycosylation (e.g., mammalian glycosylating or deglycosylating enzymes). Also included are sequences which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, phosphothronine, as well as sequences which have been modified to improve their resistance to proteolytic degradation or to optimize solubility properties.

[0023] The invention also includes antisense sequence of the sequence encoding LYC4 polypeptide. Said antisense sequence can be used to inhibit expression of LYC4 in cells.

[0024] The invention also include probes, typically having 8-100, preferably 15-50 consecutive nucleotides. These

probes can be used to detect the presence of nucleic acid molecules coding for LYC4 in samples.

[0025] The present invention also includes methods for detecting LYC4 nucleotide sequences, which comprises hybridizing said probes to samples, and detecting the binding of the probes. Preferably, the samples are products of PCR amplification. The primers in PCR amplification correspond to coding sequence of LYC4 polypeptide and are located at both ends or in the middle of the coding sequence. In general, the length of the primers is 20 to 50 nucleotides.

[0026] A variety of vectors known in the art, such as those commercially available, are useful in the invention.

[0027] In the invention, the term "host cells" includes prokaryotic and eukaryotic cells. The common prokaryotic host cells include Escherichi coli, Bacillus subtilis, and so on. The common eukaryotic host cells include yeast cells, insect cells, and mammalian cells. Preferably, the host cells are eukaryotic cells, e.g., CHO cells, COS cells, and the like.

[0028] In another aspect, the invention also includes antibodies, preferably monoclonal antibodies, which are specific for polypeptides encoded by LYC4 DNA or fragments thereof. By "specificity" is meant an antibody which binds to the LYC4 gene products or a fragments thereof. Preferably, the antibody binds to the LYC4 gene products or a fragments thereof and does not substantially recognize and bind to other antigenically unrelated molecules. Antibodies which bind to LYC4 and block LYC4 protein and those which do not affect the LYC4 function are included in the invention. The invention also includes antibodies which bind to the LYC4 gene product in its unmodified as well as modified form.

[0029] The present invention includes not only intact monoclonal or polyclonal antibodies, but also immunologically-active antibody fragments, e.g., a Fab' or $(Fab)_2$ fragment, an antibody light chain, an antibody heavy chain, a genetically engineered single chain Fv molecule (Lander, et al.,US Pat No. 4,946,778), or a chimeric antibody, e.g., an antibody which contains the binding specificity of a murine antibody, but the remaining portion of which is of human origin.

[0030] The antibodies in the present invention can be prepared by various techniques known to those skilled in the art. For example, purified LYC4 gene products, or its antigenic fragments can be administrated to animals to induce the production of polyclonal antibodies. Similarly, cells expressing LYC4 or its antigenic fragments can be used to immunize animals to produce antibodies. Antibodies of the invention can be monoclonal antibodies which can be prepared by using hybridoma technique (See Kohler, et al., Nature, 256; 495,1975; Kohler, et al., Eur. J. Immunol. 6: 511,1976; Kohler, et al., Eur. J. Immunol. 6: 292, 1976; Hammerling, et al., In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, N.Y., 1981). Antibodies of the invention comprise those which block LYC4 function and those which do not affect LYC4 function. Antibodies in the invention can be produced by routine immunology techniques and using fragments or functional regions of LYC4 gene product. These fragments and functional regions can be prepared by recombinant methods or synthesized by a polypeptide synthesizer. Antibodies binding to unmodified LYC4 gene product can be produced by immunizing animals with gene products produced by prokaryotic cells (e.g., E. coli); antibodies binding to post-translationally modified forms thereof can be acquired by immunizing animals with gene products produced by eukaryotic cells (e.g., yeast or insect cells).

[0031] The full length human LYC4 nucleotide sequence or its fragment of the invention can be prepared by PCR amplification, recombinant method and synthetic method. For PCR amplification, one can obtain said sequences by designing primers based on the nucleotide sequence disclosed in the invention, especially the sequence of ORF, and using cDNA library commercially available or prepared by routine techniques known in the art as a template. When the sequence is long, it is usually necessary to perform two or more PCR amplifications and link the amplified fragments together in the correct order.

[0032] Once the sequence is obtained, a great amount of the sequences can be produced by recombinant methods. Usually, said sequence is cloned in a vector which is transformed into a host cell. Then the sequence is isolated from the amplified host cells using conventional techniques.

[0033] In addition to recombinant techniques, the protein fragments of the invention may also be prepared by direct chemical synthesis using solid phase synthesis techniques (Stewart et al., (1969) Solid-Phase Peptide Synthesis, WH Freeman Co., San Francisco; Merrifield J. (1963), J. Am. Chem. Assoc. 85: 2149-2154). In vitro protein synthesis can be performed manually or automatically, e.g., using a Model 431 Peptide Synthesizer (Applied Biosystems, Foster City, CA). The fragments of protein of the invention can be synthesized separately and linked together using chemical methods so as to produce full-length molecule.

[0034] The sequences encoding the protein of the present invention are also valuable for gene mapping. For example, the accurate chromosome mapping can be performed by hybridizing cDNA clones to a chromosome in metaphase. This technique can use cDNA as short as about 500bp, or as long as about 2000bp, or more. For details, see Verma et al., Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York (1988).

[0035] Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found in, e.g., Mendelian Inheritance in Man (available on-line through Johns Hopkins University Welch Medical Library). The relationships between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis.

[0036] Then, the differences in the cDNA or genomic sequence between affected and unaffected individuals can also be determined. If a mutation is observed in some or all of the affected individuals but not in any normal individual, then the mutation is likely to be the causative agent of the disease.

[0037] The substances which act with the LYC4, e.g., receptors, inhibitors and antagonists, can be screened out by various conventional techniques and using the protein of the invention.

[0038] The protein, antibody, inhibitor, antagonist or receptor of the invention provide different effects when administrated in therapy. Usually, these substances are formulated with a non-toxic, inert and pharmaceutically acceptable aqueous carrier. The pH typically ranges from 5 to 8, preferably about 6-8, although pH may alter according to the property of the formulated substances and the diseases to be treated. The formulated pharmaceutical composition is administrated in conventional routine including, but not be limited to, intramuscular, intraperitoneal, subcutaneous, intracutaneous, or topical administration.

[0039] As an example, the human LYC4 protein of the invention may be administrated together with the suitable and pharmaceutically acceptable carrier. The examples of carriers include, but are not limited to, saline, buffer solution, glucose, water, glycerin, or the combination thereof. The pharmaceutical formulation should be suitable for the delivery method. The human LYC4 protein of the invention may be in the form of injections which are made by conventional methods and using physiological saline or other aqueous solution containing glucose or auxiliary substances. The pharmaceutical compositions in the form of tablet or capsule may be prepared by routine methods. The pharmaceutical compositions, e.g., injections, solutions, tablets, and capsules, should be manufactured under sterile conditions. The active ingredient is administrated in therapeutically effective amount, e.g., from about lug to 5mg per kg body weight per day. Moreover, the polypeptide of the invention can be administrated together with other therapeutic agent.

[0040] When the human LYC4 polypeptides of the invention are used as a pharmaceutical, the therapeutically effective amount of the polypeptides are administrated to mammals. Typically, the therapeutically effective amount is at least about 10 ug/kg body weight and less than about 8 mg/kg body weight in most cases, and preferably about 10ug-1mg/kg body weight. Of course, the precise amount will depend upon the factors, such as delivery methods, the subject health, and the like, and is within the judgment of the skilled clinician.

[0041] In one embodiment, the polynucleotide of the invention is 775 bp in full length whose detailed sequence is shown in SEQ ID NO: 3 with the ORF located at positions 179-619. Said polynucleotide was obtained as follows: human brain λ gt11 cDNA library (Clontech) was used as a template and PCR was carried out with the synthetic forward primer A1 : 5'-CACACGTCGAAGCTGCTGCAGTG-3' and reverse primer B1 :5'-CATCCTGCATCCCCGGATGAAGC-3'. A target fragment of 775bp was obtained. The sequencing of the PCR product gave the full length cDNA sequence shown in SEQ ID NO: 3.

[0042] Homology comparison showed that the nucleotide sequence and the coded protein sequence of the invention shared remarkable homology to other lysozymes from different origins. Therefore, it indicates it is a new member of lysozyme family and has some important functions of the family.

[0043] Lysozyme can lyse cells by hydrolyze the beta(1-4) glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylgluconic acid (NAG) of the bacterial cell wall. In the organisms, lysozyme can act as a nonspecific immune molecule against bacterial infections, and as a digestive enzyme in enteron and some mollusks which live on bacteria. Further, lysozyme has the function of inhibiting tumor growth. In 1955, Caselli and Shumacher (Boll Ocul 34:513-533, 1955) reported on the lysozyme-mediated 70% inhibition of neoplastic transformation in cornea of chicken infected by Rous sarcoma virus. Many other experiments indicated that lysozyme participates in the process of tumor diffusion and interacts with phospho- and glucolipid molecule of tumor cells. The inhibition effect on human tumor of lysozyme was reported and patented (1980 Jpn Kokai, Tokkyo Koho 33,409; 1980 Jpn Kokai Tokkyo Koho 33,408). As to the mechanism of lysozyme inhibition on tumor, there are two possibilities: (1) lysozyme directly activates the organism's immunity functions; (2) lysozyme indirectly enhances the organism's immune ability (1989 Anticancer Research 9, 583-592).

Description of Drawings

[0044] Fig. 1 shows an alignment comparison of amino acid sequences of human LYC4 and other lysozymes. Fig. 1A shows a homology comparison of amino acid sequences of human LYC4 and lysozyme C of *Reeves's pheasant* (sp|p24533). Fig. 1B shows a homology comparison of amino acid sequences of human LYC4 and lysozyme C of green pheasant (sp|p49663). The identical amino acids are indicated by ":" between the sequences, and the similar amino acids indicated by ".". The similar amino acids are as follows: A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W.

[0045] The invention is further illustrated by the following examples. It is appreciated that these examples are only intended to illustrate the invention, but not to limit the scope of the invention. For the experimental methods in the following examples, they are performed under routine conditions, e.g., those described by Sambrook. et al., in Molecule Clone: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press, 1989, or as instructed by the manufacturers, unless otherwise specified.

Examples

Example 1

The cloning and sequencing of LYC4 cDNA sequence

1. Amplification with primers

[0046] The template was human brain X gt 11 cDNA library (commercially available from Clontech). PCR with forward primer A1 : 5'-CACACGTCGAAGCTGCTGCAGTG-3' (SEQ ID NO: 1) and reverse primer B1:5'-CATCCTGCATCCCCG-GATGAAGC-3' (SEQ ID NO: 2) was carried out. The PCR condition for A1/B was 4 mins at 93°C; followed by 35 cycles with 1 min at 93°C, 1 min at 70°C, and 1 min at 72°C; and, finally 5 mins at 72°C. The PCR fragments were detected by electrophoresis. The target fragment was 775bp.

2. Sequencing PCR products

[0047] The obtained PCR products were linked with pGEM-T ® vector (Promega) and transformed into E. coli JM103. The plasmids were extracted using QIAprep Plasmid Kit (QIAGEN). The oriented serial deletion of the inserted fragments was carried out with Double-Stranded Nested Deletion Kit (Pharmacia), and the deletants were quickly identified by PCR and arranged in order. The deletants successively cut-off were sequenced with SequiTherm EXCEL™ DNA Sequencing Kit (Epicentre Technologies). A full length cDNA sequence of 775bp was obtained by overlapping the sequences with computer software. The detailed sequence is shown in SEQ ID NO: 3 with an open reading frame (ORF) located at nucleotides 179-619.

[0048] According to the resultant full-length cDNA sequence, the amino acid sequence of LYC4 was deduced, having 146 amino acid residues totally. See SEQ ID NO: 4 for its amino acid sequence in details.

Example 2

Homologous comparison

[0049] The full length cDNA sequence of LYC4 and the coded protein were used for homologous screening Non-redundant GenBank + EMBL + DDBJ + PDB and GenBank CDS translations + PDB + SwissProt + Spupdate + PIR databases by BLAST algorithm. The result showed that they shared high homology to other members of the lysozyme family. The amino acid sequence of LYC4 shares 43.4% identity and 56.6% similarity with *Reeves's pheasant* lysozyme C (splp24533) (Fig. 1A), and 42.3% identity and 56.1% similarity with lysozyme C of green pheasant (sp|p49663) (Fig. 1B), when analyzed by PCGENE software.

[0050] In particular, in amino acid sequence of LYC4, there exists a 19 amino acids signature sequence of lysozyme and alpha-lactoalbumin: $CX_3CX_2(L/M/F)X_3(D/E/N)(L/I)X_5C$ [Note: In the sequence, X represents any amino acid, digits such as "2" denote the number of amino acid, "(L/M/H)" represents any of these three amino acids]. Lysozyme and alpha-lactoalbumin are two proteins related closely in evolution (Eur. J. Biochem. 182: 111-118). In the protein of the present invention, the sequence matching the signature is: CHMSCSALLNPNLEKTIKC(residues 92-110 in SEQ ID NO: 4). It indicates that the LYC4 of the present invention belongs to lysozyme family, and has the relative functions of the lysozyme family.

[0051] The human LYC4 protein has a signal peptide of 19 amino acids (residues 1-19 of SEQ ID NO: 4). After cleavage of the signal peptide, the mature human LYC4 protein has the amino acid sequence of residues 20-146 of SEQ ID NO: 4.

[0052] Lysozyme can lyse cells by hydrolyze the beta(1-4) glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylgluconic acid (NAG) of the bacterial cell wall. In the organisms, lysozyme can act as a nonspecific immune molecule against bacterial infections, and as a digestive enzyme in enteron and some mollusks which live on bacteria.

[0053] Lysozyme has important applications in both industry and medicine.

[0054] First, in industry (mainly in food industry), lysozyme can be used as a preservative or additive for food. In this respect, the Japanese have developed many use of lysozyme and owe many patents. For example, they use lysozyme as a preservative for fresh fruit, vegetable, soybean milk, marine foods and meat. Lysozyme can also be used as an additive for infant's foods to simulate human milk (1988, Crit Rev Food Sci Nutr 26(4):359-395).

[0055] In respect of pharmaceutical use, lysozyme can be used to cure viral and bacterial infections. For example, EDTA-tris-lysozyme solutions are effective on the pseudomonas cystitis induced by E.coli infection. Lysozyme concentration in human and animal serum is an indicator of infection. Zajaczkowska-Bialowas and Murai studied the relationship between lysozyme activity in saliva and diseases of oral cavity. The result showed that lysozyme had obvious alleviation effect on the symptom of chronic periodontitis. Besides, they found the synergistic effects of lysozyme and some anti-

biotics. When lysozyme was used alone, even in a large amount, the bacteriolysis effect on *S. aureus* was little. But with the presence of amoxicillin, the lysis effect was enhanced and in proportion to the amount of lysozyme (1988 Crit Rev Food Sci Nutr 26(4):359-395).

[0056] Further, lysozyme has the function of inhibiting tumor growth. In 1955, Caselli and Shumacher (1955, Boll Ocul 34:513-533) reported on the lysozyme-mediated 70% inhibition of neoplastic transformation in cornea of chicken infected by Rous sarcoma virus. Many other experiments indicated that lysozyme had some relationship to the inhibition of tumor diffusion (1988 Clin. Expl. Metastasis 6:245-253; 1998 Folia Onclo 10, Suppl A: 219-224; 1988 Eur. J. Cancer Clin. Onco. 124:1737-1743). It is also found that lysozyme interacts with phospho- and glucolipid molecule of tumor cells. The lysozyme's inhibition effect on human tumor was reported. Laterza successfully cured a case of small intestine reticulation sarcoma with diffusion after operation and radiotherapy ("Atti del II Simposium Intemazionale sul Lisozima ", Milano. 7-8-9 1961. Vol I, sez V, pp 49-50). Battaglia et al. found that, though lysozyme could not reduce the volume of tumor, it had distinct effects of pain-killing and helping recovery in curing carcinomas of stomach, prostate, uterus and mammary gland ("Atti del II Simposium Internaionale sul Lisozima di Fleming", Milano. 3-4-5 1964. Vol I, sez IV, pp 69-76). In Japan, the application of lysozyme in curing cancer was patented (1980 Jpn Kokai Tokkyo Koho 33, 409; 1980 Jpn Kokai Tokkyo Koho 33,408). Besides, A. Vacca et al. in 1985 reported an attempt of curing multiple myeloma by chemoimmunology with oral lysozyme as an immunomodulating agent. Their experiments indicated that 50% of the patients treated with a large amount of lysozyme had improved immune ability as compared with the controls (Chemiother IV n.2:147-155,1985). As to the mechanism of lysozyme inhibition on tumor, there are two possibilities: (1) lysozyme directly activates the organism's immunity functions; (2) lysozyme indirectly enhances the organism's immune ability (1989 Anticancer Research 9, 583-592).

Example 3

Expression of LYC4 in E. coli

[0057] The cDNA sequence encoding LYC4 was amplified with oligonucleotide PCR primers corresponding to 5'- and 3'-end of said DNA sequence, using human brain λ gt 11 cDNA library (Clontech) as a template. The resultant product was used as an insertion fragment.

[0058] The sequence of 5'-end oligonucleotide primer was:
5'-TTGCGGATCCATGAAGGCATCCGTGGTTC-3' (SEQ ID NO: 5).

[0059] This primer contained a cleavage site of restriction endonuclease BamH I, followed by 19 nucleotides of LYC4 coding sequence starting from the start codon.

[0060] The sequence of 3'-end primer was:
5'-GTTCGTCGACCTACAGCTTGCAACCATCC-3'(SEQ ID NO: 6).

[0061] This primer contains a cleavage site of restriction endonuclease SalI, a translation terminator and partial LYC4 coding sequence.

[0062] These cleavage sites of restriction endonuclease in primers corresponded to the cleavage sites in bacterial expression vector pQE-9 (Qiagen Inc., Chatsworth, CA). Vector pQE-9 encodes an antibiotic resistance (Amp'), a bacterial replication origin (ori), an IPTG-adjustable promotor/operon (P/O), a ribosome-binding site (RBS), a six-hisitine tag (6-His) and cloning sites of restriction endonuclease.

[0063] Vector pQE-9 and insertion fragments were digested by BamHI and SalI, and then linked together, ensuring that the open reading frame started from the bacterial RBS. Then, the linkage mixture was used to transform E.coli M15/rep4 (Qiagen) containing multi-copy of plasmid pREP4 which expressed repressor of lacI and was resistant to kanamycin (Kan'). Transformants were screened out in LB medium containing Amp and Kan. The positive clones of transformant were cultured overnight in LB liquid medium supplemented with Amp (100ug/ml) and Kan (25ug/ml). The plasmids were extracted. The size and direction of the inserted fragments were verified by HindIII digestion. The sequencing confirmed that LYC4 cDNA fragment was correctly inserted into the vector.

[0064] The overnight culture was 1:100-1:250 diluted, inoculated into large volume medium, and cultured until the 600nm optical density (OD_{600}) reached 0.4-0.6. IPTG (isopropylthio-beta-D-galactoside) was added to final concentration of 1mM. By deactivating repressor of LacI, IPTG induced and promoted P/O, thereby increasing the expression of gene. The cells were cultured for another 3-4 hours, and then centrifuged (6000Xg, 20 mins). The cultures were sonicated, and cell lysate was collected and diluted with 6M guanidine hydrochloride. After clarification, the dissolved LYC4 in solution were purified by nickel-chelated column chromatography under the conditions suitable for the tight binding of 6-His tagged protein and column. LYC4 was eluted with 6M-guanidine hydrochloride (pH 5.0). The denaturalized proteins in guanidine hydrochloride were precipitated by several methods. First, guanidine hydrochloride was separated by dialysis. Alternatively, the purified protein, which was isolated from nickel-chelated column, bound to the second column with decreased linear gradient of guanidine hydrochloride. The proteins were denatured when binding to the column, and then eluted with guanidine hydrochloride (pH 5.0). Finally, the soluble proteins were dialyzed with PBS, then preserved

in glycerol stock solution with the final glycerol concentration of 10% (w/v).

[0065] The molecular weight of the expressed protein was 16 kDa, as identified by 12% SDS-PAGE.

[0066] Moreover, the sequencing results of the 10 amino acids at the N- and C-terminal of the expressed protein indicated that they were identical to those in SEQ ID NO: 4.

Example 4

Expression of LYC4 in eukaryotic cells (CHO cell line)

[0067] In this example, the cDNA sequence encoding LYC4 was amplified with oligonucleotide PCR primers corresponding to 5'- and 3'-end of said DNA sequence, using human brain λ gt 11 cDNA library (Clontech) as a template. The resultant product was used as an insertion fragment.

[0068] The sequence of 5'-end oligonucleotide primer was:
5'-TTGCAAGCTTATGAAGGCATCCGTGGTTC-3'(SEQ ID NO: 7).

[0069] This primer contained a cleavage site of restriction endonuclease HindIII, followed by 20 nucleotides of LYC4 coding sequence starting from the start codon.

[0070] The sequence of 3'-end primer was:
5'-GTTCGGATCCCTACAGCTTGCAACCATCC-3' (SEQ ID NO: 8)

[0071] The primer contained a cleavage site of restriction endonuclease BamHI, a translation stop codon, and partial LYC4 coding sequence.

[0072] These cleavage sites of restriction endonuclease in primers corresponded to the cleavage sites in expression vector pcDNA3 for CHO cell. This vector encoded two kinds of antibiotic resistance (Amp' and Neo'), a phage replication origin (fl ori), a virus replication origin (SV40 ori), a T7 promoter, a virus promoter (P-CMV), a Sp6 promoter, a polyadenylation signal of SV40 and the corresponding polyA sequence thereof, a polyadenylation signal of BGH and the poly A sequence thereof.

[0073] The vector pcDNA3 and insertion fragment were digested with HindIII and BamHI, and linked together. Subsequently, E.coli strand DH5 a was transformed with linkage mixture. Transformants were screened out in LB medium containing Amp. The clones containing the needed constructs were cultured overnight in LB liquid medium supplemented with Amp (100 ug/ml). Plasmids were extracted. The sequencing indicated that LYC4 cDNA fragment was correctly inserted into the vector.

[0074] Plasmids were transfected into CHO cells by lipofection with Lipofectin Kit (GIBco Life). After transfecting the cells for 48 hours and screening the cells with G418 for 2-3 weeks, the cells and cell supernatant were collected and the enzyme activity of the expressed protein was measured. G418 was removed and the transformants were subcultured continuously. The mixed clonal cells were limiting diluted and the subclones with higher protein activity were selected. The positive subclones were mass cultured by routine methods. 48 hours later, the cells and supernatant were collected. The cells were ultrasonicated. Using 50mM Tris-HCl (pH7.6) solution containing 0.05% Triton as an equilibrium solution and eluent, the active peek of the protein was collected with a pre-balanced Superdex G-75 column. Then, using 50mM Tris-HCl (pH8.0) solution containing 0-1 M NaCl as an eluent, the protein was gradiently washed on a DEAE-Sepharose column balanced with 50mM Tris-HCl (pH8.0) solution. The active peek of the protein was collected. The solution of the expressed protein was dialyzed with PBS (pH7.4), and finally lyophilized and preserved.

[0075] The molecular weight of the expressed protein was about 15 kDa as identified by 12% SDS-PAGE.

[0076] Meanwhile, a 5'-end oligonucleotide primer was designed to remove signal peptide: 5'-TTGCAAGCTT TACATCTTGG GGCGTTG-3' (SEQ ID NO: 9). The above procedure of Example 4 was repeated. An expressed protein with the molecular weight of 15 kDa was obtained, which was LYC4 without the signal peptide.

[0077] Moreover, the sequencing results of the 10 amino acids at the N- and C-terminal of the expressed protein indicated that they were identical to those in SEQ ID NO: 4.

Example 5

Antibody preparation

[0078] Antibodies were produced by immunizing animals with the recombinant proteins obtained in the above examples. The method was as follows: the recombinant proteins were isolated by chromatography, and stored for use. Alternatively, the protein was isolated by SDS-PAGE electrophoresis, and obtained by cutting eletrophoretic bands from gel. The protein was emulsified with Freund's complete adjuvant of the same volume. The emulsified protein was injected intraperitoneally into mice at a dosage of 50-100ug/0.2ml. 14 days later, the same antigen was emulsified with Freund's incomplete adjuvant and injected intraperitoneally into mice at a dosage of 50-100ug/0.2ml for booster immunization. Booster immunization was carried out every 14 days, for at least three times. The specific activity of the obtained

antiserum was evaluated by its ability of precipitating the translation product of LYC4 gene in vitro.

[0079] Further, it is appreciated that, in the above teaching of the invention, the skilled in the art can make certain changes or modifications to the invention, and these equivalents are still within the scope of the invention defined by the appended claims of the present application.

SEQUENCE LISTING

[0080]

(2) INFORMATION FOR SEQ ID NO: 1:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 23bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear

(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 1:
CACACGTCGA AGCTGCTGCA GTG 23

(2) INFORMATION FOR SEQ ID NO: 2:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 23bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear

(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 2:
CATCCTGCAT CCCCGGATGA AGC 23

(2) INFORMATION FOR SEQ ID NO: 3:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 775bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: double
(D)TOPOLOGY: linear

(ii)MOLECULAR TYPE: cDNA
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
   1 CACACGTCGA AGCTGCTGCA GTGGCTGTTC TTCCCCTCCC CTTGGAAAGG CACCAAAAGC
  61 CAGCCTTTGG AAGGGCTGGT GGACTGTGCC CTTCTTTCCC TTCAGAGAAC CAGTGTCCCT
 121 GTGACTCCAC CCACTCATCT GGCCACCGTT GCCCTGACCT GCCAGGAGCC TGGAGAAGAT
 181 GAAGGCATCC GTGGTTCTCT CCCTCCTTGG CTACCTGGTG GTTCCAAGTG GTGCTTACAT
 241 CTTGGGGCGT TGCACAGTGG CTAAGAAACT CCACGATGGA GGCCTGGATT ATTTTGAGGG
 301 CTATAGCCTT GAGAACTGGG TGTGCCTGGC CTACTTTGAG AGCAAGTTCA ACCCCATGGC
 361 CATCTACGAG AACACACGTG AGGGCTACAC TGGCTTTGGC CTCTTTCAGA TGCGTGGCAG
 421 TGACTGGTGT GGCGACCATG GCAGGAACCG CTGCCATATG TCATGTTCCG CTTTACTGAA
 481 TCCTAATTTA GAGAAGACAA TTAAATGTGC CAAGACCATT GTAAAAGGAA AAGAAGGGAT
 541 GGGAGCATGG CCCACCTGGT CCCGGTACTG CCAGTACTCC GATACCCTGG CACGGTGGCT
 601 GGATGGTTGC AAGCTGTAGC CGCCTGCATG GCCCCTGCAG CACTCACCAG TTGCATCTTG
 661 TGAATGAAGG TGCTTTTCTG CTTGCTGCTT CAGTCAATCC TTTTGATGAT CTCACCACTT
 721 TAAGAGTTCC AGATGGAAAA AGACAAAAGT TTGCTTCATC CGGGGATGCA GGATG
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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 146 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: lineal

(ii) MOLECULE TYPE: polypeptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
  1 Met Lys Ala Ser Val Val Leu Ser Leu Leu Gly Tyr Leu Val Val
 16 Pro Ser Gly Ala Tyr Ile Leu Gly Arg Cys Thr Val Ala Lys Lys
 31 Leu His Asp Gly Gly Leu Asp Tyr Phe Glu Gly Tyr Ser Leu Glu
 46 Asn Trp Val Cys Leu Ala Tyr Phe Glu Ser Lys Phe Asn Pro Met
 61 Ala Ile Tyr Glu Asn Thr Arg Glu Gly Tyr Thr Gly Phe Gly Leu

 76 Phe Gln Met Arg Gly Ser Asp Trp Cys Gly Asp His Gly Arg Asn
 91 Arg Cys His Met Ser Cys Ser Ala Leu Leu Asn Pro Asn Leu Glu
106 Lys Thr Ile Lys Cys Ala Lys Thr Ile Val Lys Gly Lys Glu Gly
121 Met Gly Ala Trp Pro Thr Trp Ser Arg Tyr Cys Gln Tyr Ser Asp
136 Thr Leu Ala Arg Trp Leu Asp Gly Cys Lys Leu
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 29bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear

(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 5:
TTGCGGATCC ATGAAGGCAT CCGTGGTTC 29

(2) INFORMATION FOR SEQ ID NO: 6:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 29bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear

(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 6:
GTTCGTCGAC CTACAGCTTG CAACCATCC 29

(2) INFORMATION FOR SEQ ID NO: 7:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 29bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear

(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 7:
TTGCAAGCTT ATGAAGGCAT CCGTGGTTC 29

(2) INFORMATION FOR SEQ ID N0: 8:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 29bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear

(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 8:
GTTCGGATCC CTACAGCTTG CAACCATCC 29

(2) INFORMATION FOR SEQ ID NO: 9:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH: 27bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear

(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 9:
TTGCAAGCTT TACATCTTGG GGCGTTG 27

Claims

1. An isolated DNA molecule comprising a nucleotide sequence encoding a polypeptide having the lysozyme activity of human LYC4 protein, said polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4 and amino acid residues 20-146 of SEQ ID NO: 4.

2. The DNA molecule of claim 1, wherein the encoded polypeptide comprises amino acid residues 20-146 of SEQ ID

NO: 4.

3. The DNA molecule of claim 1, comprising nucleotides 179-619 of SEQ ID NO: 3.

4. An isolated LYC4 polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4 and amino acid residues 20-146 of SEQ ID NO: 4.

5. The polypeptide of claim 4, comprising amino acid residues 20-146 of SEQ ID NO: 4.

6. A vector containing the DNA molecule of claim 1.

7. A host cell transformed by the vector of claim 6.

8. The host cell of claim 7, wherein it comprises E.coli.

9. The host cell of claim 7, wherein it comprises a eukaryotic cell.

10. A method for producing a polypeptide having the lysozyme activity of human LYC4 protein, the method comprising the steps of:

(a) forming an expression vector comprising the nucleotide sequence encoding the polypeptide having the activity of LYC4 protein, wherein said nucleotide sequence is operably linked with an expression regulatory sequences, and wherein said polypeptide has an amino acid sequence selected from the group consisting of SEQ ID NO: 4 and amino acid residues 20-146 of SEQ ID NO: 4;
(b) introducing the vector of step (a) into a host cell, thereby forming a recombinant cell of LYC4 protein;
(c) culturing the recombinant cell of step (b) under the conditions suitable for expression of LYC4 polypeptides;
(d) isolating the polypeptides having the activity of LYC4 protein.

11. The method of claim 10, wherein the nucleotide sequence comprises nucleotides 179-619 of SEQ ID NO: 3.

12. A nucleotide molecule-comprising the antisense sequence of the DNA molecule of claim 1, wherein the antisense sequence is completely complementary to the DNA molecule of claim 1.

Patentansprüche

1. Ein isoliertes DNA-Molekül, das eine Nukleotid-Sequenz umfasst, die für ein Polypeptid kodiert, dass die Lysozym-Aktivität von humanem LYC4 Protein hat, wobei das Polypeptid eine Aminosäuresequenz umfasst, die aus der Gruppe, die aus SEQ ID Nr. 4 und den Aminosäureresten 20-146 der SEQ ID Nr. 4 besteht, ausgewählt wird.

2. Das DNA-Molekül nach Anspruch 1, wobei das kodierte Polypeptid die Aminosäurereste 20-146 der SEQ ID Nr. 4 umfasst.

3. Das DNA-Molekül nach Anspruch 1, das die Nukleotide 179-619 der SEQ ID Nr. 3 umfasst.

4. Ein isoliertes LYC4 Polypeptid, das eine Aminosäuresequenz umfasst, die aus der Gruppe, die aus SEQ ID Nr. 4 und den Aminosäureresten 20-146 der SEQ ID Nr. 4 besteht, ausgewählt wird.

5. Das Polypeptid nach Anspruch 4, dass die Aminosäurereste 20-146 der SEQ ID Nr. 4 umfasst.

6. Ein Vektor, der das DNA-Molekül nach Anspruch 1 umfasst.

7. Eine Wirtszelle, die durch den Vektor nach Anspruch 6 transformiert wurde.

8. Die Wirtszelle nach Anspruch 7, wobei sie *E. coli* umfasst.

9. Die Wirtszelle nach Anspruch 7, wobei sie eine eukaryotische Zelle umfasst.

10. Ein Verfahren zur Herstellung eines Polypeptids, das die Lysozym-Aktivität des humanen LYC4 Proteins hat, wobei das Verfahren die Schritte umfasst:

(a) Bilden eines Exprimierungsvektors, der die Nukleotid-Sequenz umfasst, die für das Polypeptid mit der Aktivität des LYC4 Proteins kodiert, wobei die Nukleotid-Sequenz in funktioneller Verbindung mit regulatorischen Exprimierungssequenzen steht, und wobei das Polypeptid eine Aminosäuresequenz hat, die aus der Gruppe, die aus der SEQ ID Nr. 4 und den Aminosäureresten 20-146 der SEQ ID Nr. 4 besteht, ausgewählt wird;
(b) Einführen des Vektors nach Schritt (a) in eine Wirtszelle, wobei dabei eine rekombinante Zelle des LYC4 Proteins gebildet wird;
(c) Kultivieren der rekombinanten Zelle nach Schritt (b) unter den Bedingungen, die für die Exprimierung der LYC4 Polypeptide geeignet ist;
(d) Isolieren der Polypeptide mit der Aktivität des LYC4 Proteins.

11. Das Verfahren nach Anspruch 10, wobei die Nukleotid-Sequenz die Nukleotide 179-619 der SEQ ID Nr. 3 umfasst.

12. Ein Nukleotid-Molekül, das die Antisense-Sequenz des DNA-Moleküls nach Anspruch 1 umfasst, wobei die Antisense-Sequenz zu dem DNA-Molekül nach Anspruch 1 vollständig komplementär ist.

Revendications

1. Molécule d'ADN isolée comprenant une séquence nucléotidique codant un polypeptide possédant l'activité lysozymique de la protéine LYC4 humaine, ledit polypeptide comprenant une séquence d'acides aminés choisie dans le groupe constitué par SEQ ID NO : 4 et les résidus d'acides aminés 20 à 146 de SEQ ID NO : 4.

2. Molécule d'ADN selon la revendication 1, dans laquelle le polypeptide codé comprend les résidus d'acides aminés 20 à 146 de SEQ ID NO:4.

3. Molécule d'ADN selon la revendication 1, qui comprend les nucléotides 179 à 619 de SEQ ID NO : 3.

4. Polypeptide LYC4 isolé comprenant une séquence d'acides aminés choisie dans le groupe constitué par SEQ ID NO : 4 et les résidus d'acides aminés 20 à 146 de SEQ ID NO : 4.

5. Polypeptide selon la revendication 4, qui comprend les résidus d'acides aminés 20 à 146 de SEQ ID NO : 4.

6. Vecteur contenant la molécule d'ADN selon la revendication 1.

7. Cellule hôte transformée par le vecteur selon la revendication 6.

8. Cellule hôte selon la revendication 7, dans laquelle elle comprend *E. coli.*

9. Cellule hôte selon la revendication 7, dans laquelle elle comprend une cellule eucaryote.

10. Procédé de production d'un polypeptide possédant l'activité lysozymique de la protéine LYC4 humaine, le procédé comprenant les étapes consistant à :

(a) former un vecteur d'expression comprenant la séquence nucléotidique codant le polypeptide possédant l'activité de la protéine LYC4, dans lequel ladite séquence nucléotidique est liée de manière fonctionnelle à une séquence de régulation de l'expression, et dans lequel ledit polypeptide a une séquence d'acides aminés choisie dans le groupe constitué par SEQ ID NO : 4 et les résidus d'acides aminés 20 à 146 de SEQ ID NO : 4 ;
(b) introduire le vecteur de l'étape (a) dans une cellule hôte, formant ainsi une cellule recombinante de la protéine LYC4 ;
(c) cultiver la cellule recombinante de l'étape (b) dans les conditions adaptées à l'expression des polypeptides LYC4 ;
(d) isoler les polypeptides ayant l'activité de la protéine LYC4.

11. Procédé selon la revendication 10, dans lequel la séquence nucléotidique comprend les nucléotides 179 à 619 de SEQ ID NO : 3.

12. Molécule de nucléotide comprenant la séquence antisens de la molécule d'ADN selon la revendication 1, dans laquelle la séquence antisens est complètement complémentaire de la molécule d'ADN selon la revendication 1.

```
                  10        20        30        40        50        60
LYC4       MKASVVLSLLGYLVVPSGAYILGRCTVAKKLHDGGLDYFEGYSLENWVCLAYFESKFNPM
                                . ::: .:  ..  ::: ..:::: :::: : :::.::
sp|P24533  K-------------------VYGRCELAAAMKRLGLDNYRGYSLGNWVCAAKFESNFNTH
                                    10        20        30        40

                   70        80            90        100       110
LYC4       AIYENTREGYTGFGLFQMRGSDWCGDH----GRNRCHMSCSALLNPNLEKTIKCAKTIVK
           :  .:: .: : .:..:. .  ::.:     .:: ::.::::::. ..  ...::: ::.
sp|P24533  ATNRNT-DGSTDYGILQINSRWWCNDGRTPGSRNLCHISCSALLSSDITASVNCAKKIVS
               50        60        70        80        90        100

           120       130       140
LYC4       GKEGMGAWPTWSRYCQYSDTLARWLDGCKL
           ..::.:: .:   :. .:. : :. ::.:
sp|P24533  DRNGMNAWVAWRNRCKGTDVNA-WIRGCRL
              110       120
```

Fig. 1A

```
                  10        20        30        40        50        60
LYC4       MKASVVLSLLGYLVVPSGAYILGRCTVAKKLHDGGLDYFEGYSLENWVCLAYFESKFNPM
           :                . ::: .:  ..  ::: ..:::: :::: : :::.::
sp|P49663  GK------------------VYGRCELAAAMKRMGLDNYRGYSLGNWVCAAKFESNFNTG
                                   10        20        30        40

               70        80            90        100       110
LYC4       AIYENTREGYTGFGLFQMRGSDWCGDHGR-----NRCHMSCSALLNPNLEKTIKCAKTIV
           :  .:: .: : .:..:. .  ::.: ::     : ::. :::::. ..  ...::: ::
sp|P49663  ATNRNT-DGSTDYGILQINSRWWCND-GRTPGSKNLCHIPCSALLSSDITASVNCAKKIV
               50        60        70        80        90        100

           120       130       140
LYC4       KGKEGMGAWPTWSRYCQYSDTLARWLDGCKL
           .  .::.:: .:..:. .: .  :. ::.:
sp|P49663  SDGDGMNAWVAWRKHCKGTD-VNVWIRGCRL
              110       120       130
```

Fig. 1B

REFERENCES CITED IN THE DESCRIPTION

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